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# OBSERVATIONS ON THE TOXIN OF *RHIZOPUS NIGRICANS*

BY ROSS AIKEN GORTNER AND A. F. BLAKESLEE

[FROM THE BIOCHEMICAL LABORATORY OF THE STATION FOR EXPERIMENTAL EVOLUTION, THE CARNEGIE INSTITUTION OF WASHINGTON]

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# OBSERVATIONS ON THE TOXIN OF *RHIZOPUS NIGRICANS*

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[*From the Biochemical Laboratory of the Station for Experimental Evolution, The Carnegie Institution of Washington*]

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## Introduction

In a preliminary note [1] we have shown that the "presssaft" from *Rhizopus nigricans* contains a powerful toxin. At that time we had not attempted to prepare the toxin in a more concentrated form and had only ascertained that an aqueous extract from 0.045 gram of the dried mould filaments or mycelium was sufficient to kill a 1.35 kilo rabbit in two minutes when the toxin was injected intravenously.

In this paper we desire to present some further observations on the chemical nature of this toxin, methods of obtaining a more concentrated preparation and the results of physiological tests. Although we believe that we have not, as yet, isolated the toxic principle in a pure state, we have obtained preparations having a lethal toxicity of approximately 1:275,000 parts of body weight when injected intravenously into rabbits. Inasmuch as one of us (G.) must of necessity abandon further work on this subject we are presenting the results more recently obtained.

## Experimental

### 1. *Physiological Tests using Extracts of the Mycelium or Aerial Filaments*

(*a*) **Intravenous Injections.**—The presence of a toxin in the "presssaft" of *Rhizopus* was noticed during a series of immunization experiments having as their aim a chemical analysis of the cause of sex in the Mucors.[2] The aerial filaments of the mould were ground with sand and the juice expressed by means of a small beef juice press and centrifuged at high speed to remove suspended solids. When 2 c.c. of such an extract is injected intravenously into the ear of a rabbit death is almost instantaneous, the animal often expiring before the injection is complete (about 5 seconds) or before the needle can be withdrawn from the vein. In such a case there are no typical symptoms accompanying death, there is rarely any struggle and only a sudden sinking of the head to one side and a convulsive twitching of the body.

When, however, only about *one tenth* of this dose is given (diluted to 2 or 3 c.c. with physiological salt solution) the symptoms are invariably the same. A few seconds after the needle is withdrawn the head sinks to one side and a violent convulsive spasm follows. This may be followed by a "typical death" (see below) or by a short rest period followed by another convulsive spasm, depending both on the size of the dosage employed and also on the degree of resistance possessed by the animal.[1] The convulsive spasms rarely last longer than 30 seconds while the rest period between may be as long as 30 minutes. During the rest periods the animal usually lies fully stretched out, the only indication of life being its regular breathing. At the close of the final spasm there is usually a short rest period of 5 to 10 seconds followed by a spasmodic cough-like movement of the diaphragm with widely opened mouth as if gasping for breath. There are usually 8 to 10 of these cough-like movements of the diaphragm, each shorter and more rapid than the one preceding. During this period the head

[1] We have observed considerable individuality in these animals; in some instances one animal would be killed almost instantly while with the same dosage another animal of like weight would live for 15–20 minutes.

is thrown backward and the chest and abdomen are distended and turgid, so much so that only by strong pressure can the wall of the abdomen be flexed. This reaction usually lasts 8 to 12 seconds and is followed by a relaxation of the rigid abdomen, protruding eyes and death. This description of a death is meant wherever a "typical death" may be referred to later. All of these symptoms may be shown within a period of 15 seconds from the time the injection is made or death may be deferred for several hours. In the latter case the behavior of the animal is usually "normal" until within a few minutes before death. Such a condition accompanies a dosage *just sufficient* to cause death.

When the dosage is *just sub-lethal* there is usually a short convulsive spasm followed by a long period (24 to 48 hours) of extreme lethargy, and complete recovery. In nearly all instances tested a fall of nearly 4° in the body temperature of the rabbit occurred during the first 12 hours after receiving the injection, and as recovery progressed the temperature gradually rose. Since the rabbit has no constant temperature it is impossible to determine the significance of this factor with exactness, but it seems probable that a fall from 102.4° to 98.5° is significant, and such a fall has been observed in several instances.

No tolerance was observed when an animal which had received a just sub-lethal dose was again given a similar injection after complete recovery. On the contrary the symptoms were slightly more violent than in the first injection.

When a *slightly* smaller dose than just sub-lethal is injected *there is usually no noticeable reaction*, the animal remaining to all appearances normal. If, however, after 30 to 60 minutes, a slight additional quantity is injected *just sufficient to raise the original dosage within the lethal limits*, there is a sudden violent reaction and death within a few seconds or minutes.

(*b*) **Subcutaneous Injections.**—Only four experiments were made where the "presssaft" was injected subcutaneously, but since each of these injections gave similar results it was thought unnecessary to prolong the investigation further. In each instance the injection was made under the skin of the back under aseptic precautions. The "presssaft" was sterilized either by rapidly heating the solution to *just boiling* and then cooling quickly or else by the addi-

tion of ether or chloroform in a sterile tube, plugging with sterile cotton and evaporating the antiseptic under diminished pressure.

A typical instance of the results is as follows: Rabbit No. 60, weight 1330 grams, received subcutaneously 6.7 c.c. of the "presssaft" at 11 A.M. May 21. This dosage was the equivalent of about 20 intravenous lethal doses. No results were observed during the first 24 hours. After several days it was noticed that the skin seemed loose and the flesh "flabby" but no other symptoms were observed. Eight days after the injection a small purulent ulcer was observed on the belly of the rabbit and the weight had fallen to 1050 grams. Although the ulcer was well washed with "peroxide" and bound up with iodoform gauze it increased in size and inasmuch as it showed no sign of healing the animal was chloroformed 12 days after making the injection. The weight at death was 850 grams.

This result would seem at first glance to be due to bacterial infection at the time the injection was made, but we do not believe this to be the case, for in numerous instances we have injected subcutaneously the "presssaft" of other moulds (as well as physiological salt solution) with far less precaution against infection and have never had such injection followed by either lesions or by a considerable loss in weight.

On examination we found that there was an open channel from the site of the sore to the point of injection and subsequent investigation in other cases showed that the "presssaft" is not absorbed (or else that serum exudes) so that the lump formed by the mass of liquid under the skin gradually works down the side of the animal until it reaches the belly. This movement of the liquid under the skin is probably brought about by the action of gravity, possibly combined with a corrosive action of the toxin. After reaching the belly the mass of liquid can be distinctly felt under the skin for 2 to 3 days, at the end of which time the skin is eaten through from within and the ulcer begins.

(*c*) **Administration of the Mould per Os.**—Rabbits, guinea pigs, and chickens were fed the moist mycelium or the dried and ground mycelium and aerial filaments of *Rhizopus*. In only one instance was there any noticeable effect. A guinea pig weighing 705 grams

was fed one gram daily of the dried mould mycelium in gelatin capsules on five consecutive days. There was a marked loss of weight each day and at the end of the sixth day the weight had fallen to 505 grams, showing a loss of nearly 30 per cent of the original body weight. Unfortunately at about this time one of the contagious diseases to which guinea pigs are subject developed in the animal house and in the course of the next 10 days all of the pigs had died. We are unable, therefore, to say with certainty that this loss in weight was due to the feeding of the mould although we believe that such may be the case, since the animal was apparently perfectly normal in its actions during the feeding experiment and also since it was one of the last of the animals to die.

Rabbits were fed upon bread upon which a heavy culture of the mould was growing and also upon corn meal upon which *Rhizopus* had been grown for a week or more. No ill effects of such feedings were observed.

### 2. *Autopsy after Death following an Intravenous Injection*

Dr. O. T. Avery of the Hoagland Laboratories, Brooklyn, was kind enough to witness the symptoms caused by an intravenous injection of the toxin into both rabbits and guinea pigs and also to assist in an autopsy. The only abnormal conditions apparent were a pronounced heart block, the auricles beating about 3:1, and a partial inflation of the lungs. Avery has investigated the effects of the toxin prepared from the tubercle bacilli by the method of Vaughan [3] as well as the toxic properties of the split products of edestin [4] and he pronounced the death symptoms and the autopsy following *Rhizopus* injection as being "typical" of toxins of this type, and practically indistinguishable from an anaphylactic intoxication. Unless we assume that all animals are born "sensitive" to *Rhizopus* it will be almost impossible to call the death anaphylaxis. On the other hand the preparation of the toxin precludes any splitting of the protein molecules such as is brought about by Vaughan's hydrolysis with alkali. It seems possible however that *Rhizopus* so utilizes the protein of the culture medium that a split product having toxic properties similar

to the toxins prepared by Vaughan's method is present in the "presssaft."

### 3. *Attempts to Isolate the Chemical Substance Responsible for the Toxic Action*

About 5 c.c. of the "presssaft" was evaporated to dryness *in vacuo* over sulphuric acid and extracted first with warm ether and then with warm alcohol. The ether and alcohol were evaporated in a dessicator and the residue taken up with physiological salt solution and injected intravenously into a rabbit. No ill effects were observed. It was later found that the toxic principle could be completely precipitated from its aqueous solution by the addition of four volumes of 95 per cent alcohol. Following this observation a concentration of the toxin was obtained by two methods.[1]

**Method.** (*a*)—The mycelium of *Rhizopus*, grown on solid agar medium under conditions of extreme moisture with a consequent nearly complete suppression of sporangial formation, was stripped

[1] Since preparing this paper for publication our attention has been called to the work of Paladino-Blandini.[5] Paladino-Blandini tested *Rhizopus*, together with other genera, for the presence of a toxic phenol by extracting the dried mycelium with 90 per cent alcohol evaporating the extract to dryness, and injecting a suspension of the dried residue. In *Rhizopus* three injections of 0.05 grams each, spaced at an interval of half an hour, were necessary to kill a 3 ko. rabbit. The death symptoms of the rabbit were very similar to those which we have observed with our toxin. We have, however, injected 0.108 gram of the alcohol soluble substances (extracted from the aerial mycelium with *absolute* alcohol in a Wiley extraction apparatus) into a 1200 gram rabbit without ill effects. It seems possible that extraction with 90 per cent alcohol may dissolve a part of our toxin, or, which seems still more probable, that some of the toxin formed a colloidal solution with the 90 per cent alcohol. This will occur whenever alcohol is added to a solution of the toxin in the absence of electrolytes. Another possible cause of death is embolism due to the very considerable quantity of insoluble lipins injected into the vein.

It is certain that Paladino-Blandini had only a small part of the toxin present in his *Rhizopus* mycelium, for we have found that the alcohol soluble material does not exceed 30 per cent of the dry weight of the fungus, therefore to obtain 0.15 gram of the alcohol soluble portion would require 0.50 gram of the dry mycelium, the aqueous extract of which would contain sufficient toxin to kill *five* 3 kilo rabbits *within two minutes* after the injection.

off, cleaned from agar, dried over sulphuric acid *in vacuo* and powdered.[1]

The powdered mould in 25 gram lots was poured into 200 c.c. of boiling water and stirred for about 30 seconds, then filtered on a Buchner funnel with suction. This process was repeated on the residue twice more with fresh hot water, the filtrates were united and concentrated at 50° to 55° under a pressure of about 30 mm. to about 200 c.c. This solution was then placed in a collodion bag and dialysed against repeated changes of distilled water, or, since tests showed that the water outside the bag contained no toxin, against running water for 72 hours, using chloroform as an antiseptic inside of the bag. The liquid in the bag was clear, straw-colored and opalescent, it frothed quite readily and dripped fairly viscid. This liquid was centrifuged at high speed to remove some flecks of solid (agar?) and was then poured into four volumes of 95 per cent alcohol. A heavy white precipitate formed at once. The precipitate was allowed to settle in a tall cylinder, the yellowish supernatant liquid was siphoned off and the white solid packed in a tube by centrifugation. The solid was stirred with fresh alcohol, again packed in a centrifuge and dried over sulphuric acid *in vacuo*. Yield 4.85 per cent from the ♀ race and 4.60 per cent from the ♂ race.[6]

**Method.** (*b*)—One hundred grams of the dried and ground mycelium was stirred into 2000 c.c. of water at the room temperature and allowed to stand with frequent stirring for two hours. The yellowish liquid was then filtered by suction, the residue pressed strongly in a large beef juice press and the liquid obtained added to the first filtrate. Three volumes of 95 per cent alcohol were then added to the united filtrates, yielding at once a copious white precipitate. The partially extracted mould was again stirred into 1000 c.c. of water and allowed to stand at room temperature for 18 hours, when the mixture was filtered by suction and the residue

[1] That a possible slight admixture of the nutrient medium with the mycelium has no significance so far as the toxin obtained is concerned, is shown by check experiments with the fungus grown on oatmeal where all possibility of contamination with the substratum was avoided by carefully cutting off for use only the free aerial mycelium. This preparation showed a toxicity which was *possibly* slightly greater than that of the mycelium from agar.

again pressed dry. This filtrate was likewise precipitated by three volumes of alcohol, yielding a small but appreciable amount of precipitate. The alcoholic solutions were united in a large vessel and allowed to settle for 48 hours, the clear yellow supernatant liquid was then siphoned off and the white solid filtered on a Buchner funnel with suction. The solid was air dried and dissolved as completely as possible in 250 c.c. of water *without the aid of heat.* A considerable quantity did not dissolve (coagulated albumen [?] or agar [?]). The solution was then centrifuged and the clear amber liquid poured into 1000 c.c. of alcohol. The precipitate was allowed to settle, the solution centrifuged and the white solid dried *in vacuo* over sulfuric acid. This solid was powdered and again dissolved in 150 c.c. of water, only a few particles remaining undissolved. The mixture was allowed to stand for 12 hours, centrifuged and poured into 1000 c.c. of alcohol. A white turbidity resulted but on standing for 24 hours no precipitate settled. This was probably caused by an almost complete removal of electrolytes from the solution, for on the addition of two drops of a saturated solution of sodium chloride a copious curdy precipitate was at once thrown down, leaving the supernatant liquid perfectly clear and colorless. This precipitate was packed in a centrifuge, dried *in vacuo* over sulphuric acid and powdered. Yield, 3.58 per cent from the ♀ race and 3.74 per cent from the ♂ race of the mould.

### 4. *Physiological Tests with the Concentrated Toxin*

(*a*) **The Intravenous Lethal Dose.** — The data for this series can be best expressed in the form of a table, the various preparations of the toxin being designated by (*a*) ♂ (prepared by method [*a*] from the ♂ race of *Rhizopus*) (*a*) ♀, (*b*) ♂, and (*b*) ♀.

(*b*) **Subcutaneous Injection.** — Only one animal was given a subcutaneous injection of the concentrated toxin. Inasmuch as we have found that sterilization with heat causes a loss of toxicity (see "Chemical Properties of the Concentrated Toxin," below), the dry powder was sterilized by placing it in a sterile vial, adding about 5 c.c. of a mixture of ether and 95 per cent alcohol, plugging the vial with sterile cotton and, after allowing the preparation to

| Toxin | Wt. injected | Vol. of injection | Wt. rabbit | Time to die | Toxin to body wt. |
|---|---|---|---|---|---|
| (*a*) ♂ | 0.0080 gr. | 8 c.c. | 1050 gr. | 50 mins. "typical" | 1: 131,250 |
| | 0.0060 gr. | 6 c.c. | 875 gr. | 3 mins. "typical" | 1: 145,800 |
| | 0.0025 gr. | 2.5 c.c. | 560 gr. | 90 secs. "typical" | 1: 224,000 |
| | 0.0040 gr. | 4 c.c. | 1000 gr. | Recovered | 1: 250,000 |
| (*a*) ♀ | 0.0080 gr. | 2 c.c. | 1000 gr. | 19 mins. "typical" | 1: 125,000 |
| | 0.0040 gr. | 1 c.c. | 1000 gr. | Recovered | 1: 250,000 |
| (*b*) ♂ | 0.0065 gr. | 3.3 c.c. | 1390 gr. | 5 mins. "typical" | 1: 215,000 |
| | 0.0050 gr. | 2.5 c.c. | 1365 gr. | Nearly normal for first 5 hrs. but found dead at 18 hrs. | 1: 273,000 |
| (*b*) ♀ | 0.0080 gr. | 4 c.c. | 1200 gr. | 60 secs. "typical" | 1: 150,000 |
| | 0.0075 gr. | 3.75 c.c. | 1510 gr. | 75 secs. "typical" | 1: 200,000 |
| | 0.0072 gr. | 3.6 c.c. | 1550 gr. | 3 mins. "typical" | 1: 215,000 |
| | 0.0070 gr. | 3.5 c.c. | 1535 gr. | 1 min. 40 secs. "typical" | 1: 220,000 |
| | 0.0065 gr. | 3.25 c.c. | 1475 gr. | 5 hrs. "typical" | 1: 225,000 |
| | 0.0036 gr. | 1.80 c.c. | 810 gr. | Not dead in 7 hrs but dead in 15 hrs. | 1: 225,000 |
| | 0.0040 gr. | 2.0 c.c. | 1480 gr. | No typical symptoms. Recovered | 1: 370,000 |

stand for several hours, evaporating the alcohol-ether in a dessicator over sulfuric acid. The dried residue was then dissolved in sterile salt solution.

Under aseptic precautions 0.10 gram of preparation (*b*) ♀, dissolved in 5 c.c. of physiological salt solution, was injected subcutaneously on the back of a rabbit weighing 1550 grams. No immediate effect was observed. Four days later, however, the weight had fallen to 1300 grams and the flesh was very flabby and soft. There was a soft lump low down on the side of the animal and immediately below the point of injection. This lump evidently contained serum or the unabsorbed liquid which was injected. No

sore had as yet opened. The following day, however (five days after the injection) there was a small ulcer at the spot where the lump had been the previous day. The sore continued to spread somewhat, but contrary to the experiments where the "presssaft" was injected, a scab formed over the sore. This scab or thickened skin area could be distinctly felt as a heavy ridge extending from the point of injection to the spot where the skin had broken through. Following the formation of the scab there was a decided improvement in the condition of the animal and 13 days after the injection the weight had risen to 1530 grams. Following this the scab became loosened, leaving the flesh dry. There was no pus formation observed. The animal increased in weight and the lesion healed normally.

(*c*) **Intraperitoneal Injection.** — Two experiments were made in which the concentrated toxin was injected into the peritoneal cavity, using preparation (*b*) ♀.

Rabbit No. 1 weighed 1440 grams and received 1.5 c.c. of a solution containing 0.06 gr. of the toxin, or 1:24,000 parts of body weight. The animal appeared normal for four hours following the injection but was found dead 12 hours later. An autopsy showed that the lower intestine was highly inflamed and that the peritoneal cavity contained a considerable quantity of a yellow fluid, the lungs were discolored with gray blotches but the other organs were normal.

Rabbit No. 2 weighted 1625 grams and received 2.5 c.c. of a solution containing 0.10 gr. of the toxin, or 1:16,250 parts of body weight. No unusual symptoms were observed for some time with the exception that the animal did not seem as active as usual. Forty-eight hours later the weight had fallen to 1450 grams, the temperature was 104°, the animal had diarrhoea and was running at the nose and the skin of the abdomen was highly inflamed. Ninety-six hours after the injection the animal was found dead, weight 1360 grams. An autopsy showed the caecum filled with a highly putrescent liver-colored mass, the stomach packed with food, while the lower intestine and the rectum contained only four or five formed feces whereas normally there is a large number. The small intestine contained a small quantity of a thin yellow fluid (probably bile) and was very much distended with gas. The gall

bladder was three or four times normal size and the liver, otherwise normal, was badly stained with bile. The spleen was much darker than normal, being almost black at the median end. The lungs were a blackish gray, badly mottled, without a trace of pink color being apparent. There was no evidence of inflammation within the body cavity.

(*d*) **Intramuscular Injection.** — Only one experiment was made where the injection was given intramuscularly, and this injection was not an intentional one. A rabbit weighing 1310 grams was given an injection of 5 c.c. of a solution containing 0.20 grams of preparation (*b*) ♀. The intention was to give the injection intraperitoneally, but later developments and the autopsy showed that the needle had not penetrated the abdominal wall and that the injection had been between the muscle plates of the abdomen. Forty-eight hours after the injection the weight had fallen to 1180 grams, the temperature was 104.9°, the skin of the abdomen was highly inflamed and very tender to the touch, but no sores were observed and the animal ate regularly. Eight days after the injection the weight had fallen to 1045 grams and a bad purulent ulcer had formed on the abdomen. The animal was chloroformed and an autopsy performed. All of the viscera were in a normal condition and only a slight inflammation was observed on the inner wall of the abdomen immediately under the ulcer. In this instance, therefore, an intramuscular injection caused the same developments as a subcutaneous injection, only the symptoms were somewhat more pronounced. This may be due to the higher dosage of toxin (30 to 40 intravenous lethal doses) employed, or it may be due to the fact that the irritation was between the muscle plates rather than subcutaneous.

(*e*) **Administration of the Concentrated Toxin per Os.** — Only one feeding experiment was made but the quantity of the toxin was sufficiently large to determine whether or not the mould would be dangerous in moderate doses.

A rabbit weight 1330 grams was fed, by means of a catheter inserted into the stomach, 50 c.c. of a solution containing 1.0 grams of preparation (*b*) ♂ — in other words 150 to 200 times the intravenous lethal dose. *This is the equivalent of 26* to *27 grams of the dried mould mycelium and of probably 100 to 125 grams of the*

*moist growing mould. It is a far larger amount than any animal could possibly procure at any one feeding under any natural conditions.* No abnormal effect has been observed to date. The weight of the animal steadily increased, the animal weighing 1590 grams 19 days after the feeding.

From this experiment, in which we are certain that no loss of the toxin occurred through vomiting, we conclude that, in rabbits at least, the toxin is not absorbed from the alimentary canal, or if absorbed it has been so changed as to produce no ill effects.

### 5. *Chemical Properties of the Concentrated Toxin*

The product obtained by either method (*a*) or (*b*) forms a yellowish white powder readily soluble in water to a straw-colored opalescent solution from which it is again precipitated by basic lead acetate, phosphotungstic acid + HCl (without the addition of the HCl only a white opalescence is produced), three or four volumes of alcohol, and by mercuric chloride. Tannic acid gives a fine white precipitate with a solution of the toxin, and Folin's "Phenol Reagent" gives a deep indigo-blue color.

The Biuret, Xanthoprotein, Adamkiewicz's, Liebermann's and Molisch's tests are all strongly positive, while Millon's test is positive but faint. The substance does not reduce Fehling's solution, but after hydrolysis with acids a strong reduction takes place. The toxicity of the solution is not altered by passing through a Berkefelt filter, but on warming with freshly ignited animal charcoal all of the toxin is absorbed by the charcoal and the solution is absolutely non-toxic. This is still the case when the solution + bone black is quickly raised to *just boiling* and then rapidly cooled.

Digestion at 37° with pepsin in 0.2 per cent HCl for three hours did not impair the toxicity, but a further digestion of this solution with trypsin in a weakly alkaline medium for 18 hours (no antiseptic) caused a loss of about 75 per cent of its activity.

The toxicity is slowly destroyed by boiling. 0.20 grams of preparation (*b*) ♂ was dissolved in 50 c.c. of physiological salt solution and boiled under a reflux condenser for three hours. There was no evidence of decomposition nor was there any precipitate formed, but on testing the physiological activity of the solution it was found that the lethal dose was about 1:37,000 parts of body

weight. This would indicate the loss of about 75 per cent of the original activity.

When, however, the toxin is boiled with N/10 HCl there is a rapid loss of activity. 0.20 grams of preparation (*b*) ♂, was boiled under a reflux condenser with 25 c.c. of N/10 HCl for $1\frac{1}{4}$ hours, 25 c.c. of N/10 NaOH was then added and the solution, which now gave a strong reduction with Fehling's solution, was poured into four volumes of 95 per cent alcohol. The white precipitate which was thrown down was collected in a centrifuge, dried, dissolved in a small volume of physiological salt solution and injected into a rabbit intravenously. The dosage was equivalent to 1:14,000 but no ill effects were observed, indicating that all or nearly all of the toxin had been destroyed.

The possibility that we were dealing with a saponine was tested by adding a strong solution of the toxin to washed blood corpuscles, but no trace of hemolysis occurred.

### *Analyses*

Nitrogen was determined on the different preparations in part by Dumas' method and in part by Kjeldahl's. From the nitrogen figures it is seen that we are not dealing with a pure compound but with a mixture, and therefore, no further analyses were attempted. From the figures for nitrogen and from the chemical tests to which the toxin responds one can postulate all sorts of classifications, such as glucoside, the split product of a protein, peptides, etc., but we are unwilling to even venture a guess as to the chemical nature of the toxin. That can only be determined when some method has been devised by which the toxic principle can be obtained in a pure condition.

Preparation (*a*) ♂:
0.1916 gr. gave 7.4 c.c. N at 24° and 768 mm. Nitrogen found = 4.43%.

Preparation (*a*) ♀:
0.2300 gr. gave 5.4 c.c. N at 22° and 768 mm., 0.4180 gr. gave 9.0 c.c. N/10 $NH_4OH$. Nitrogen found = 2.69% and 3.01%.

Preparation (*b*) ♂:
0.2554 gr. gave 10.9 c.c. N at 20.5° and 762 mm., 0.2260 gr. gave 9.6 c.c. N at 21.5° and 768 mm. Nitrogen found = 4.88% and 4.87%.

Preparation (*b*) ♀:
0.2977 gr. gave 10.25 c.c. N/10 $NH_4OH$, 0.2253 gr. gave 9.0 c.c. N at 23° and 767 mm. Nitrogen found = 4.82% and 4.54%.

### 6. *Evidences of a Toxin in Other Species of the* Mucorineae

During a series of injections we have obtained some evidence that there may be a small amount of a toxic substance in the "presssaft" of other moulds, such as "*Mucor V*" and *Phycomyces nitens*. We have made a large number of intravenous injections of the "presssaft" of these species and have observed that where a non-lethal dose is increased in an injection three to four days later death may occur. In two sets of experiments we have lost four out of five rabbits injected with the ♀ race of *Mucor V* while none of the animals receiving an equal quantity of the "presssaft" from the ♂ race have died. It was also observed that whereas 2 c.c. of the "presssaft" from the filaments of *Phycomyces* caused no untoward symptoms, that an injection of 3 c.c often caused convulsions and sometimes death. An attempt was therefore made to concentrate the toxin from *Phycomyces*, if such a toxin existed.

Twenty grams of the dry aerial filaments of the ♂ race of *Phycomyces nitens* was treated as in method (*a*) (page 358). The alcohol precipitate was dark green and the powder on drying was almost black. A solution of the dried preparation was too dark to permit of the observation of color tests. A solution of the powder did not reduce Fehling's solution, but after acid hydrolysis a strong reduction was observed. A yield of 2.60 per cent of the dried product was obtained.

In physiological tests no effect was observed when an intravenous injection of 1:15,000 was given. This preparation has therefor little or no toxic properties.

The fact that *Phycomyces* gives a yield of 2.60 per cent of a non-toxic substance by the same method that *Rhizopus* yields 4.60 and 4.85 per cent of a toxic substance confirms to our mind the fact that our toxin is far from being a pure compound. By the method of preparation glycogen, or glyco-proteins, would, if present, be precipitated with the toxin and would account for the Molisch's and Fehling's tests.

## Summary

1. *Rhizopus nigricans* contains a powerful toxin which is water soluble and which can be completely precipitated from its aqueous solution by three to four volumes of 95 per cent alcohol.

2. A solution of the toxin is non-dialyzable, gives the protein tests as well as Molisch's test, but reduces Fehling's solution only after a previous hydrolysis with acid. The toxin is completely absorbed from its aqueous solution by warming with bone black, is slowly destroyed by boiling its aqueous solution and rapidly destroyed by boiling with N/10 HCl. Peptic digestion for three hours is without effect on the toxicity.

3. In intravenous injection into rabbits the lethal dose of our preparations lies between 1:225,000 and 1:275,000 parts of body weight.

4. In subcutaneous and intramuscular injections the toxin causes the formation of purulent ulcers.

5. In intraperitoneal injections there is some evidence that paralysis of the digestive tract ensues.

6. Administered *per os* in large doses there was no harmful effect observed.

7. Inasmuch as a 2.6 per cent yield of a *non-toxic* substance having similar chemical reactions can be prepared from *Phycomyces nitens*, and inasmuch as the nitrogen content of our toxic preparations varies from 2.8 per cent to 4.9 per cent, we believe that our preparations are, in all probability, highly impure, perhaps containing 50 per cent or more of inactive impurities, so that the actual intravenous lethal dose of the pure toxin is probably much higher than has been observed, possibly near the extreme toxicity of 1:500,000.

## Literature

[1] Blakeslee and Gortner: Biochemical Bulletin, 1913, ii, p. 542.

[2] Blakeslee and Gortner: See C. B. Davenport, "The Department of Experimental Evolution," Year Book of the Carnegie Institution of Washington, 1913, xii, p. 99.

[3] White and Avery: Journal of Medical Research, 1912, n.s. xxi, p. 317.

[4] White and Avery: Journal of Infectious Diseases, 1913, xiii, p. 103.

[5] Paladino-Blandini: Arch. Farm. Sperimentale e Sci. Affini., 1906, v, p. 606.

[6] Blakeslee: Science, n.s. xxxvii, p. 880.

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